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# NADH dehydrogenase in *Neurospora crassa* contains myristic acid covalently linked to the ND5 subunit peptide

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**Abstract**

The mitochondrial, proton-pumping NADH:ubiquinone oxidoreductase consists of at least 35 subunits whose synthesis is divided between the cytosol and mitochondria; this complex I catalyzes the first steps of mitochondrial electron transfer and proton translocation. Radiolabel from [$^3$H]myristic acid was incorporated by *Neurospora crassa* into the mitochondrial-encoded, ~70 kDa ND5 subunit of NADH dehydrogenase, as shown by immunoprecipitation. This myristate apparently was linked to the peptide through an amide linkage at an invariant lysine residue (Lys546), based upon analyses of proteolysis products. The myristoylated lysine residue occurs in the predicted transmembrane helix 17 (residues 539–563) of ND5. A consensus amino acid sequence around this conserved residue exists in homologous subunits of NADH dehydrogenase. Cytochrome *c* oxidase subunit 1, in all prokaryotes and eukaryotes, contains this same consensus sequence surrounding the lysine which is myristoylated in *N. crassa*. 

*Keywords:* NADH dehydrogenase; Myristoylation; Mitochondria; (*Neurospora crassa*)

## 1. Introduction

The mitochondrial proton-pumping NADH dehydrogenase (NADH:ubiquinone oxidoreductase; EC 1.6.99.3), as the first step in electron transport, couples the transfer of electrons from NADH to ubiquinone with the translocation of protons across the mitochondrial inner membrane, establishing a proton motive force required for ATP synthesis [1]. This NADH dehydrogenase (or complex I) is structurally the most complicated of the proton-translocating respiratory chain complexes of mitochondria, with at least 35 different subunits. In *Neurospora crassa*, seven of the subunits are encoded by mitochondrial genes and synthesized on mitochondrial ribosomes, with the remaining (approximately) 28 subunits encoded by nuclear genes, synthesized in the cytoplasm, and imported into mitochondria [2]. These subunits are assembled into an L-shaped structure in which one arm is buried within the membrane and the other protrudes into the mitochondrial matrix [3,4], and the two arms are assembled independently before joining to form the mature complex [5]. The mitochondrial-encoded subunits, which are evolutionarily conserved, are localized in the membrane-intrinsic arm of this assembly [6], whereas the matrix arm contains most of the prosthetic groups involved in redox reactions [4]. *Saccharomyces cerevisiae* does

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not produce this enzyme complex [7], and most of our understanding of the contributions of individual nuclear-encoded subunit peptides to assembly and function of the complex has come from gene disruption studies in *N. crassa* [8–11]. In the absence of these subunits, complex I assembly is defective and the vegetative cells exhibit reduced growth rate. Our awareness of the importance of mitochondrial-encoded subunits to normal mitochondrial function has come from analysis of mutations of these genes associated with human degenerative diseases [12], mutations selected in mouse fibroblast lines [13], and in *Neurospora* strains mutant in mitochondrial genes [14]. In human cells, the absence of the mtDNA-encoded subunit ND4 led to a failure to assemble other mtDNA-encoded subunits and to a complete loss of NADH-dependent respiration and NADH:$Q_1$ oxidoreductase activity, while at least some of the nuclear-encoded subunits in the peripheral arm assembled normally, permitting a normal NADH:$Fe(CN)_6$ oxidoreductase activity [15]. In mouse cells the loss of the ND6 subunit peptide disrupted assembly of the membrane arm of the enzyme and enzyme activity [13]. The assembly of the membrane domain, but not the peripheral arm, of complex I was impaired in the E35 stopper mutant of *N. crassa* that is deleted in mitochondrial ND2 and ND3 subunits of the enzyme [14].

Earlier, we observed [16,17] that when we labeled *N. crassa* cells with [$^{14}$C]pantothenic acid, the radioactivity was incorporated into a protein which later proved [18] to be a mitochondrial acyl carrier protein (ACP). This ACP also is a subunit of the peripheral arm of complex I [19], and we have found that it coprecipitates with cytochrome *c* oxidase [16,17]. It seemed to us that this association of the ACP with these mitochondrial proteins might indicate a functional relationship, perhaps for transfer of an acyl moiety to modify these proteins [17]. Although we have not tested this hypothesis directly, when we radiolabeled *N. crassa* cells with [$^3$H]myristic acid we found that radioactivity was stably incorporated into about five proteins of isolated mitochondria (N. Plesofsky, R. Brambl, unpublished results). Further study showed that one of the proteins that contained this radiolabel specifically from [$^3$H]myristic acid was the mitochondrial-encoded subunit 1 of cytochrome *c* oxidase [20]. The myristoylation of this highly conserved protein occurred at an internal, invariant lysine [20] that is within predicted transmembrane helix VIII, a helix which comprises part of a pore proposed either for delivery of substrate protons to the site where oxygen is reduced to water or for transport of protons across the membrane [21,22].

In the present study, we found that [$^3$H]myristic acid is incorporated into a peptide subunit of complex I in *N. crassa*. The synthesis of this peptide is insensitive to cycloheximide, it has an apparent molecular mass of the peptide product of the mitochondrial gene ND5, and proteolytic digestion of this protein, radiolabeled with [$^3$H]myristate, yields a pattern indicating a modification of a specific lysine within the ND5 subunit. This modified lysine is within a 14-residue amino acid sequence that is very similar to the sequence surrounding a myristoylated lysine in subunit 1 of *N. crassa* cytochrome *c* oxidase.

## 2. Materials and methods

### 2.1. Cell strains and growth

Conidia of *N. crassa* wild-type strain 74*A* or the mutant strain, *cel* (deficient in fatty acid synthase; Fungal Genetics Stock Center No. 819), were produced as described previously [23], except that the *cel* mutant was grown on medium supplemented with Tween-40 or Tween-80 (1% v/v, polyoxyethylenesorbitan mono-palmitate or -oleate) [24]. Conidia (1 mg/ml) were germinated at 30°C in 2% sucrose-minimal salts medium for 7 h as described earlier [25] and radiolabeled as described [20] for 1 h with [$^3$H]myristate, [$^3$H]palmitate, or [$^{35}$S]methionine (100 μCi/100 ml); the *cel* mutant was supplemented with Tween-40 or Tween-80 for radiolabeling with myristate or palmitate, respectively. After 60 min, an excess (500- to 1000-fold) of unlabeled methionine was added to those cultures radiolabeled with [$^{35}$S]methionine. All cultures were harvested rapidly by cold, vacuum filtration, rinsed with cold mitochondrial extraction buffer, and frozen at −80°C. To identify protein products of mitochondrial ribosome origin, conidia were germinated for 5.5 h and they were treated with cycloheximide (100 μg/ml) to inhibit cytosolic ribosome protein synthesis. Two minutes later [$^{35}$S]methionine (100 μCi/100 ml) was

added to these cells and they were incubated an additional 30 min, before being processed as described above.

### 2.2. Immunoprecipitation analyses

The mitochondrial fraction, prepared as described elsewhere [25], was used for immunoprecipitation analysis. Immunoprecipitation experiments were performed with an antiserum to the *Neurospora* multiple-subunit cytochrome *c* oxidase [26] and with an antiserum to the 20.8 kDa subunit of *Neurospora* NADH dehydrogenase that is able to coprecipitate the entire complex I [27,28]. The mitochondria were lysed with detergent [26], and Protein A-Sepharose (Pharmacia Biotech) coupled to IgG via dimethylpimelimidate [29] was used to isolate specific mitochondrial proteins. Protein A-Sepharose–antigen complexes were allowed to form for 4 h at 4°C, and were then washed as described [30]. Proteins were eluted with electrophoresis sample buffer and subjected to sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gel electrophoresis and fluorography [25].

### 2.3. Proteolytic analyses

We labeled cells with [$^3$H]myristate, prepared submitochondrial particles [31] and solubilized them for electrophoresis. We excised that region of the gel that appeared to contain only subunit ND5 of NADH dehydrogenase, guided by reversible staining [32] with Coomassie blue. An in-gel digestion [33] was performed with proteases (Boehringer Mannheim), Arg-C and Glu-C (*Staphylococcus* V-8 protease), and the peptides were separated in a Tris/Tricine electrophoresis system [34] with cylindrical gels. Following electrophoresis of these digests, the 10-cm gel rods were mechanically sliced (1-mm$\times$100) for analysis by liquid scintillation spectrometry [26].

### 2.4. Characterization of chemical linkage

Samples of submitochondrial particles labeled with [$^3$H]myristate or [$^3$H]palmitate were treated with methanolic KOH (1 M in 20% methanol), with Tris–HCl (1 M, pH 8.0), or with protein sample solubilization buffer [35], centrifuged at 100 000$\times g_{av}$ for 30 min, and subjected to SDS–polyacrylamide gel electrophoresis.

## 3. Results

### 3.1. Incorporation of radiolabel into mitochondrial proteins

Cells of *Neurospora* (*cel*) were labeled with [$^3$H]myristic acid; mitochondria from these cells were isolated and the proteins were electrophoretically separated in a SDS polyacrylamide gel. Fig. 1 shows at least two major proteins of these mitochondria that incorporated the label from [$^3$H]myristate. Previously, we noted that there were about five proteins in submitochondrial particles of *Neurospora* that incorporated label from [$^3$H]myristate, and we identified one of these (Fig. 1) as subunit 1 of cytochrome *c* oxidase [20].

We found that an antiserum to the NADH dehydrogenase [27,28] precipitated one protein of about 68 000 Da that had incorporated label from [$^3$H]myristate (Fig. 1). Comparison with an NADH

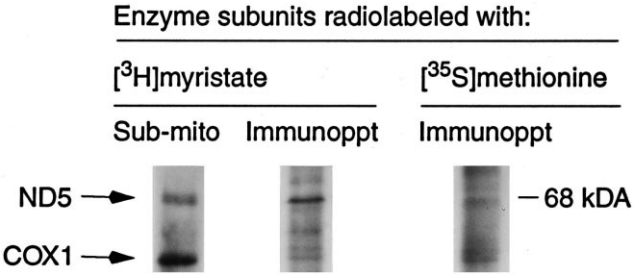


Fig. 1. Incorporation of radiolabel from [$^3$H]myristate or [$^{35}$S]methionine into submitochondrial membranes or into specific immunoprecipitates. Radiolabeled proteins from mitochondria of germinating spores were treated with antisera prepared against *Neurospora crassa* NADH dehydrogenase. The first electrophoretic lane shows the radiolabel in total protein of submitochondrial particles, and the other lanes show the immunoprecipitates from lysates of the radiolabeled mitochondria. (Only proteins migrating more slowly than 38 000 $M_r$ are shown here.) The germinating spores were radiolabeled with the fatty acid or amino acid, the submitochondrial particles were prepared and subjected directly to electrophoresis in a SDS–polyacrylamide gel or lysed with detergent and treated with antiserum, with the immunoprecipitate then subjected to electrophoresis. Treatments with antiserum to the subunit 9 of the mitochondrial ATPase or a preimmune antiserum gave no [$^3$H]myristate-labeled bands.

dehydrogenase immunoprecipitate, in which the subunits were labeled with [$^{35}$S]methionine, showed that the [$^{3}$H]myristate-labeled protein migrated at the same position as [$^{35}$S]methionine-labeled subunit ND5 (Fig. 1), a subunit encoded by the mitochondrial genome. As first reported by others [36], we observed that this protein contained radiolabel incorporated from [$^{35}$S]methionine following a 2-min pretreatment with cycloheximide, an inhibitor of cytoplasmic, but not mitochondrial, protein synthesis. A similar analysis of [$^{3}$H]palmitate-labeled mitochondrial proteins gave no indication that this fatty acid was present in any of the immunoprecipitated NADH dehydrogenase peptide subunits (data not shown). Reaction of these [$^{3}$H]myristate-labeled proteins with either a preimmune antiserum or an antiserum against subunit 9 of the mitochondrial ATPase [31] precipitated no detectable radiolabel from this fatty acid, as shown previously [20].

### 3.2. Characterization of the chemical linkage

We prepared submitochondrial particles from cells labeled with [$^{3}$H]myristate to characterize the linkage between the myristate-derived moiety and the ND5 peptide. Extracts of these submitochondrial particles were treated with methanolic KOH or Tris–HCl buffer [35]. The treatments with KOH should hydrolyze ester linkages but not amide linkages. The peptide identified as NADH dehydrogenase subunit ND5 retained its [$^{3}$H]myristate-derived label under these conditions (Fig. 2), indicating that the moiety is incorporated into the peptide in an amide linkage, as has been reported for most other cellular proteins that are modified with myristic acid. In parallel, as a control, we labeled cells with [$^{3}$H]palmitate, prepared both submitochondrial particles and microsomes, and treated them with the same reagents, followed by electrophoresis. As expected for palmitic acid,

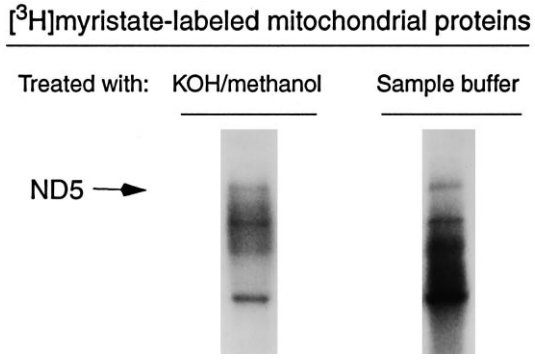


Fig. 2. Electrophoresis of [$^{3}$H]myristate-labeled ND5 subunit of NADH dehydrogenase following treatment of submitochondrial particle protein with methanolic KOH or with sample buffer (control).

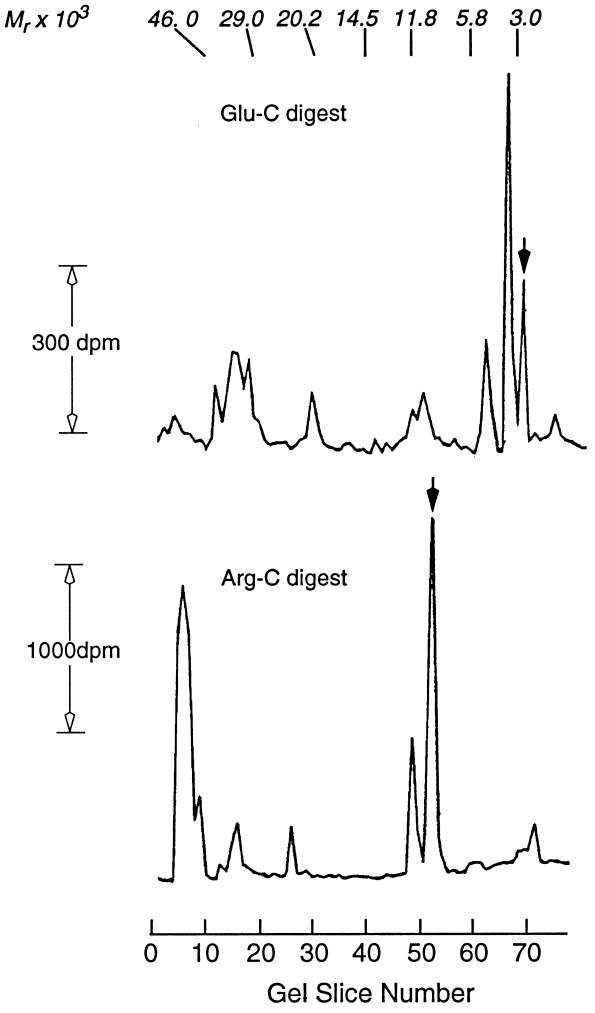


Fig. 3. Electrophoresis of [$^{3}$H]myristate-labeled ND5 subunit of NADH dehydrogenase following proteolytic hydrolysis. The Coomassie-stained ~70 kDa peptide of the initial separation gel, corresponding to the ND5 subunit peptide, was excised from the gel and subjected to proteolysis, and the products were separated by electrophoresis (direction of migration from left to right) through cylindrical gels which were frozen and sliced mechanically. Individual 1-mm slices of the electrophoretic gel were analyzed by liquid scintillation spectrometry and plotted. Proteolytic reagents were the enzymes Arg-C and Glu-C.

whose characterized linkages to proteins are esterifications, this label was lost from the proteins after treatment with methanolic KOH.

### 3.3. Identification of myristoylated lysine residue

To determine the amino acid residue of the ND5 subunit peptide that was modified by myristic acid, we subjected the peptide, labeled with [$^3$H]myristate, to site-specific hydrolysis by two proteases. Since the sequence of this subunit peptide from *N. crassa* is known [37], we expected that a proteolysis map of the [$^3$H]myristate-labeled peptide fragments, sized by electrophoresis, would allow us to identify a likely site for modification.

Glu-C (*Staphylococcus* V8) protease cuts at the carboxy side of glutamic acid residues. Complete digestion of the ND5 subunit peptide with this enzyme should yield 20 peptides, 13 of which contain lysine and range in size from 14.3 kDa to 1.3 kDa (Fig. 3). We expected that the smallest labeled peptide would represent the product of complete, rather than partial proteolytic digestion. The smallest fragment we found that contained label from [$^3$H]myristate was about 3.0 $M_r$ (Fig. 3). A predicted fragment of 3.0 kDa (peptide 14 from the N-terminus, residues 539–565) contains one lysine that is a candidate for modification. Two additional peptides that contain one or more lysines, peptide 19 (2.8 kDa) and peptide 16 (3.2 kDa), are close in size to the 3.0 $M_r$ peak and could also be candidates for myristoylation.

Complete digestion of the ND5 peptide with Arg-C protease, which cuts at the carboxy end of arginine residues, should yield 13 peptides, seven of which contain lysine and range in size from 13.8 to 3.4 kDa (Fig. 3). The smallest fragment we found that contained label from [$^3$H]myristate was 10.5 $M_r$ (Fig. 3). A predicted fragment of 11.0 kDa (fragment 11 from the N-terminus, residues 481–578) contains four lysines, one of which is also contained within fragment 14 of the Glu-C digestion. This identifies the single Lys546 within the Glu-C peptide 14 as the likely site of myristate modification. Two other, slightly larger peaks of 3.5 $M_r$ and 6.2 $M_r$ likely resulted from incomplete proteolysis. The 3.5 $M_r$ peak would contain both peptide 14 and peptide 13 (0.72 kDa). The 6.2 $M_r$ peak would contain peptide 14 and peptide 15 (3.5 kDa); with a longer Glu-C digestion, the 6.2 $M_r$ peak disappeared.

The results of these proteolysis experiments, analyzed in Fig. 4, show that the two proteolytic enzymes, Glu-C, and Arg-C, produce [$^3$H]myristate-labeled fragments of sizes that correspond to unique predicted digestion fragments of the ND5 subunit peptide. These two proteolytic digests, therefore, point to Lys546 of the ND5 peptide as the probable site of modification by [$^3$H]myristic acid. The modified Lys546 of the ND5 subunit is within and near the beginning of a very hydrophobic domain (residues 539–563) predicted to form a transmembrane helix, according to MEMbrane Structure And Top-

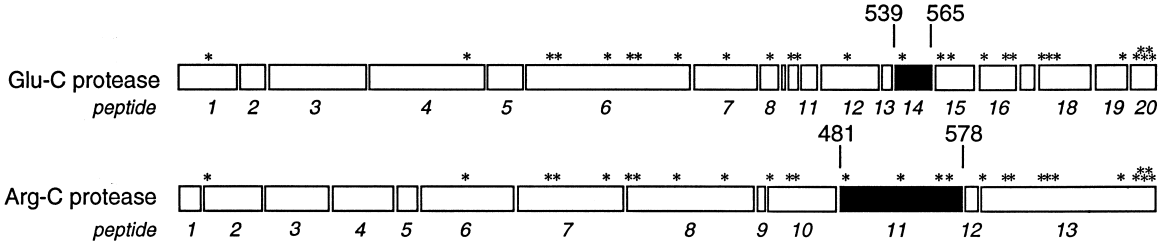


Fig. 4. Analysis of proteolytic digestion of the ND5 subunit peptide radiolabeled with [$^3$H]myristic acid, showing the deduced lysine residue modified by this fatty acid. Proteolytic reagents were the enzymes Arg-C and Glu-C. Asterisks denote lysine residues. The boxes indicate numbered proteolysis fragments generated by each of these enzymes, and filled boxes denote unique peptides in each digest that contained radioactivity from [$^3$H]myristic acid and that contained the common Lys546 residue modified with myristic acid.

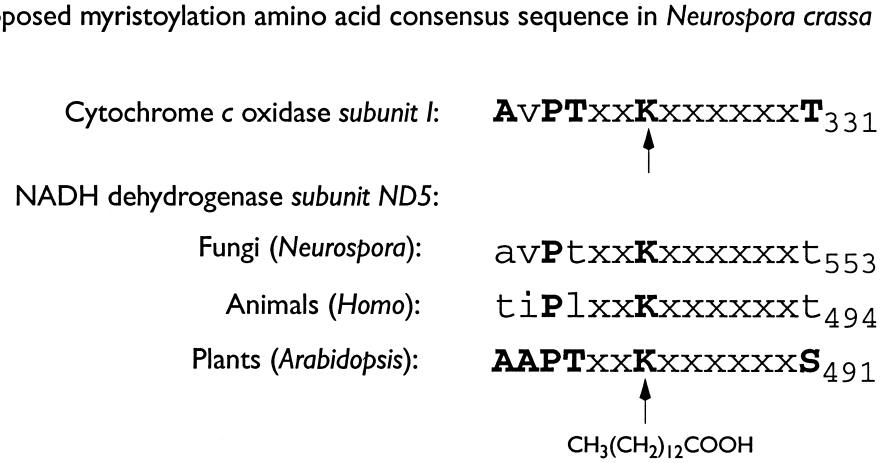


Fig. 5. Proposed amino acid myristoylation consensus sequence common to NADH dehydrogenase ND5 and cytochrome *c* oxidase subunit 1 subunit peptides in *N. crassa*, both of which are mitochondrial-encoded. Shown here are matching amino acid sequences surrounding the modified lysine of the cytochrome *c* oxidase subunit 1 [40] and the ND5 subunit of NADH dehydrogenase [37] of *N. crassa*, along with aligned sequences from the ND5 subunits of representative animal [41] and plant [42] species. Boldface, uppercase letters indicate tightly conserved residues within the biological group.

ology prediction program (version 1.5, ©D.T. Jones; London, 1993).

### 3.4. Identification of a consensus sequence for myristoylation

We examined the amino acid sequence around the ND5 subunit lysine residue that is modified with myristic acid, and we determined that within a 14-residue amino acid sequence around the myristoylated Lys546 there are highly conserved residues, for subunits of known sequence that are homologous to the ND5 subunit peptide (Fig. 5). The sequence surrounding the putative myristoylation site of the ND5 subunit is highly conserved among all the plant species, and less so for fungi and animals. However, in all cases the ND5 Lys546 (using the *N. crassa* sequence numbering) is conserved, along with the Pro542 and a hydrophobic residue at position 541; frequently there is a hydroxylated amino acid at position 553. In this analysis, we also found a nearly identical pattern within the 14-residue amino acid sequence around the myristoylated Lys324 of subunit 1 of cytochrome *c* oxidase of *Neurospora* (Fig. 5), and this consensus sequence is highly conserved in the homologous subunits of cytochrome *c* oxidases of all organisms examined, including bacteria and mammals.

## 4. Discussion

Previously, we found that the most conserved lysine of the highly conserved subunit 1 of cytochrome *c* oxidase in *Neurospora* is myristoylated [20], and in this present study we found this same modification of another mitochondrial-encoded peptide subunit, ND5, of complex I, the proton-pumping NADH dehydrogenase of the electron transport chain. The modification of this subunit is at a highly conserved lysine residue and, like that of cytochrome *c* oxidase subunit 1, the myristate modification is within a predicted transmembrane helix.

The most common type of myristoylation of proteins synthesized in the cytosol occurs cotranslationally at the α-amino group of an N-terminal glycine [38]. More rarely, myristate is esterified to an internal residue [38]. The type of internal amide linkage of mitochondrial proteins that we describe here is rare, and the mechanism of modification may be unique to these organelle enzyme subunit peptides. Although myristate is a relatively rare fatty acid in cells, it has been recently found that mitochondria possess a distinct fatty acid synthesis system and that hydroxylated myristic acid is a major product [39].

The function of myristoylation of these mitochondrial-encoded components of two complexes in the

mitochondrial electron transport chain is unknown. In the case of the cytochrome *c* oxidase, the modification occurs within a predicted helix, which contains residues that are important for delivering substrate protons to the site where oxygen is reduced to water and also for transporting protons across the membrane [21]. Replacement of this cytochrome *c* oxidase lysine residue with methionine or arginine in *Rhodobacter sphaeroides* or *Escherichia coli* abolished enzyme catalytic activity [21]. It is not yet known whether this myristoylation is involved in normal function of the proton pore of either of these two enzyme complexes, or if this modification might participate in assembly of the complex or in modulating catalytic activity in a reversible manner.

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